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## Digestibility of $\beta$ -lactoglobulin following cross-linking by *Trametes versicolor* laccase and apple polyphenols

ZIYAD TANTOUSH, LUKA MIHAJLOVIĆ<sup>#</sup>, BOJANA KRAVIĆ<sup>#</sup>, JANA  
OGNJENović<sup>#</sup>, RATKO M. JANKOV<sup>#</sup>, TANJA ČIRKOVIĆ VELIČKOVIĆ<sup>#</sup>  
and DRAGANA STANIĆ-VUČINIĆ<sup>\*\*</sup>

Faculty of Chemistry, University of Belgrade, Studentski trg 16, P. O. Box 158,  
11001 Belgrade, Serbia

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**Abstract:**  $\beta$ -Lactoglobulin (BLG) is an important nutrient of dairy products and an important allergen in cow's milk allergy. The aim of this study was to investigate the potential of laccase to cross-link BLG in the presence of an apple phenolic extract (APE) and to characterize the obtained products for their digestibility by pepsin and pancreatin. The composition of the apple phenolics used for cross-linking was determined by liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS). The apple phenolic extract contained significant amounts of quercetin glycosides, catechins and chlorogenic acid. The laccase cross-linked BLG in the presence of apple phenolics. The polymerization rendered the protein insoluble in the reaction mixture. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the cross-linking reaction mixture revealed a heterogeneous mixture of high molecular masses (cross-linked BLG), with a fraction of the BLG remaining monomeric. Enzymatic processing of BLG by laccase and apple polyphenols as mediators can decrease the biphasal pepsin–pancreatin digestibility of the monomeric and cross-linked protein, thus decreasing its nutritional value. In addition, reduced BLG digestibility can decrease its allergenic potential. Apple polyphenols can find usage in the creation of new, more functional food products, designed to prevent obesity and hypersensitivity-related disorders.

**Keywords:** apple polyphenols;  $\beta$ -lactoglobulin; laccase; allergen; digestibility.

### INTRODUCTION

Enzymatic processing of food proteins for improvement of food texture and functionality is a good replacement for chemical additives. Laccase is a polyphenol oxidase that acts on phenolic compounds and can facilitate the cross-linking

\*Corresponding author. E-mail: dstanic@chem.bg.ac.rs

<sup>#</sup> Serbian Chemical Society member.

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of proteins.<sup>1–3</sup> The cross-linking of tyrosine-containing peptides by laccase has been shown to proceed *via* tyrosyl radicals that form primarily isodityrosine, and also a small amount of dityrosine bonds.<sup>4,5</sup> The formation of disulfide bonds by the oxidation of cysteines into cystine is another way of cross-linking proteins, which laccase can induce in flour dough using ferulic acid as a mediator.<sup>3</sup> Enzymatic cross-linking of food proteins can have beneficial effect on their safety and can influence their digestibility by digestive enzymes.<sup>6</sup> Reductions of allergenicity were shown for beta-casein cross-linked by laccase/caffeic acid and tyrosinase/caffeic acid,<sup>7</sup> peanut proteins cross-linked by polyphenol oxidase,<sup>8</sup> roasted peanut allergens treated with peroxidase<sup>9</sup> and major cherry allergen exposed to tyrosinase in the presence of various polyphenols.<sup>10</sup> Thus, the usage of enzymes was anticipated as a novel approach for the production of hypoallergenic foods.<sup>11</sup>

$\beta$ -Lactoglobulin (BLG) accounts for approximately 10–15 % of the total milk proteins.<sup>12,13</sup> It also represents a serious health risk in cow's milk allergic patients as it is known as one of the major milk allergens<sup>14–16</sup> and is one of the most resistant proteins to enzymatic digestion by pepsin.<sup>17</sup> Under physiological conditions, it is a very soluble protein, thereby easily passing the transepithelial barrier and is able to cause systematic anaphylactic reactions.<sup>18</sup> It was recently shown that enzymatic processing by laccase and the employment of sour cherry phenolic extract as the mediator of enzymatic reaction may improve BLG safety and availability of peptides following digestion by pepsin, while conserving its bioactivity.<sup>19</sup>

Apples contain significant levels of polyphenols, possessing strong antioxidative<sup>20</sup> and potent immunomodulatory activity.<sup>21</sup> Recently, plant polyphenols have been defined as one class of gammadelta T cell agonist and appear to preferentially activate the mucosal T cell population.<sup>22</sup>

In this study, the cross-linking potential of laccase on purified BLG in the presence of an apple polyphenol extract (APE), as a source of phenolic mediators, and the digestibility of cross-linked BLG under simulated conditions of the gastrointestinal tract were investigated.

## EXPERIMENTAL

### Chemicals

Pepsin (from porcine stomach mucosa, 2650 U mg<sup>-1</sup> solid), pancreatin (from porcine pancreas), Folin–Ciocalteu's phenol reagent, gallic acid, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and laccase (from *Trametes versicolor*, 30.6 U mg<sup>-1</sup> of solid) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Anti-rabbit IgG labeled with alkaline phosphatase was also from Sigma. Urea and disodium hydrogen phosphate (analysis grade) were purchased from Merck (Darmstadt, Germany). The deionized water (DW) used in the experiments was purified in a Milli-Q system (Millipore, Molsheim, France).

#### *Purification of $\beta$ -lactoglobulin and determination of protein*

BLG was purified from raw milk essentially as described previously.<sup>23</sup> The purity of the protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The concentration of the BLG was determined by spectrophotometry at 280 nm ( $\epsilon = 0.943 \text{ mg}^{-1} \text{ mL cm}^{-1}$ ).

#### *Preparation and characterization of the apple phenolics*

Apple peel extract, 250 mg, (Applepoly, Apple Poly™, USA) was dissolved in 5 mL of HPLC grade ethanol, centrifuged for 15 min at 13400 rpm, and the supernatant was submitted to LC–MS analysis of the APE composition. The components of apple extract were identified according to their  $m/z$  values, UV/Vis absorption spectra and retention times. Relative quantities of phenolics were calculated from the peak areas on the chromatograms. The total phenolics in the APE were determined by spectrophotometric analysis using Folin–Ciocalteu's reagent<sup>24</sup> and are expressed as mg gallic acid equivalents (GAE)/100 mL of APE.

#### *LC–MS analysis*

Chromatographic separation was performed on Agilent 1100 series high-performance liquid chromatography–electrospray ionization–mass spectrometry–time of flight (HPLC–ESI–MS–TOF), equipped with a diode array detector (DAD). The separations were realized on a Zorbax C18 1.7  $\mu\text{m}$  column (4.6 mm $\times$ 50mm). The mobile phase consisted of water containing 0.1 % formic acid (A) and acetonitrile containing 0.1 % formic acid (B). The separation was performed on a linear gradient of acetonitrile in water (0–90 % B) during 20 min. The injection volume for all samples was 1  $\mu\text{L}$ . Mass detection was performed on an Agilent 6520 TOF detector coupled to the chromatographic system. The mass spectra were obtained at a mass-to-charge ratio ( $m/z$ ) scan range from 100 to 3200. The following MS parameters were used for the analysis: charging voltage, 2000 V; capillary voltage, 4000 V; nebulizer pressure, 45 psig; drying gas flow rate, 12 L  $\text{min}^{-1}$ . The samples were analyzed in the negative mode. Data was processed using Analyst QS 1.1 from Applied Biosystems and Chemspider database for the structure search.

#### *Polymerization of apple phenolics by Trametes versicolor laccase*

In order to obtain polymeric catechins, apple phenolics were treated with laccase. Laccase activity was determined using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) as the substrate.<sup>3</sup> 20  $\mu\text{L}$  of laccase (0.1mg  $\text{mL}^{-1}$  final concentration) was added to 430  $\mu\text{L}$  of apple phenolics extract (50 mg GAE  $\text{mL}^{-1}$ ) and 50  $\mu\text{L}$  of 200 mM acetic buffer pH 4.5. After incubation for 24 h at 37 °C, the mixture was centrifuged for 15 min at 13400 rpm and supernatant was submitted to LC–MS analysis.

#### *Electrophoresis*

SDS–PAGE was realized using a Hoefer Scientific Instrumentation apparatus (Amersham Biosciences, Uppsala, Sweden) with a discontinuous buffer system. The Protein components were resolved on 12 and 10 % polyacrylamide gels and stained using Coomassie Brilliant Blue R-250 (Sigma–Aldrich).

#### *Cross-linking of BLG by laccase in the presence of APE*

To prepare the laccase-treated samples, 1.6 mg  $\text{mL}^{-1}$  BLG, APE in the range of phenol concentration (0.06 to 0.99 mg  $\text{mL}^{-1}$  of gallic acid equivalents) and 30.95 U  $\text{mL}^{-1}$  laccase in 40 mM acetate buffer, pH 4.5 were incubated for 2 h at 37 °C with shaking and the reaction was stopped by addition of 1 mM ethylenediaminetetraacetic acid (EDTA) in final and the sample was immediately frozen. The control samples were prepared in the same manner but

the laccase and mediator were omitted. For digestibility analysis, cross-linked BLG was prepared using 0.33 mg mL<sup>-1</sup> of gallic acid equivalents APE, 30.95 U mL<sup>-1</sup> laccase and 1.6 mg mL<sup>-1</sup> BLG in a reaction mixture of 0.6 mL. After cross-linking, the reaction mixture was centrifuged for 30 min at 12340 rpm. The pellet was washed with 50 mM acetate buffer pH 4.5 two times and pellet was resuspended in 0.6 mL of 50 mM acetate buffer.

*Digestion of the cross-linked BLG by pepsin and pancreatin*

*In vitro* gastric fluid digestion of BLG was performed as described previously,<sup>23</sup> with some modifications. Briefly, fifty microliters of BLG control and laccase-treated BLG (1.6 mg mL<sup>-1</sup>), with or without APE solutions (0.24 mg mL<sup>-1</sup> of GAE), were diluted with 10 µL of DW, warmed to 37 °C, and 20 µL of pre-warmed 0.4 M HCl containing 8 g L<sup>-1</sup> of NaCl and 1.28 g L<sup>-1</sup> of pepsin were added (1 U of pepsin/µg of protein). Digestion proceeded at 37 °C with continuous shaking for 18 h. The digestion was stopped with 5 µL of 2 M Na<sub>2</sub>CO<sub>3</sub>, and samples were vortexed vigorously and mixed with 21.25 µL of five times concentrated sample buffer for SDS–PAGE analysis. Thirteen microliters of each sample were applied per lane (10 µg of BLG, laccase-treated BLG or laccase/APE treated BLG per well). A pepsin control was set up in the same manner but with no addition of BLG. BLG, laccase-treated BLG and laccase/APE treated BLG controls were prepared without pepsin. For bi-phasal pepsin–pancreatin digestion, pepsin digestion was performed as described above and the digestion was stopped with 5 µL of 2 M Na<sub>2</sub>CO<sub>3</sub>. To 85 µL of the reaction mixture, 9.5 µL of pancreatin (10 mg mL<sup>-1</sup>) in ten times concentrated phosphate buffered saline (PBS) was added and digestion proceeded for 4 h. An aliquot of 17 µL was diluted with 46 µL of water to make 63 µL of mixture for analysis, which was centrifuged at 13400 rpm for 20 min. Both supernatant and pellet samples (re-suspended in 32 µL of PBS) were mixed with five times concentrated sample buffer (16 and 8 µL, respectively) for SDS–PAGE analysis and the reaction of pancreatin digestion was stopped by boiling at 95 °C for 5 min.

## RESULTS AND DISCUSSION

The main constituents of APE are chlorogenic acid, phloretine glucosides and mono-, di- and trimeric catechines (Fig. 1A, Table I). The composition of APE used in this study was similar to previously described phenolic contents of polyphenols extracted from apple peels, although the number of components was reduced because of extensive purification.<sup>20</sup> Exposing APE to laccase induced polymerization of the polyphenol compounds (Fig. 1A) giving extensive precipitate that could not be analyzed. The soluble fraction of APE treated with laccase was subjected to LC–MS analysis and the components identified are presented in Fig. 1B and Table I. It can be observed that the spectra of the untreated and laccase-treated APE are very similar. The only significant difference is the reduction in the (–)epicatechin content after treatment. The percent (–)epicatechin decreased from 25.74 to 14.17 % of the total phenolics after laccase treatment. A very slight increase in the concentration of procyanidine C (epicatechin trimer) was also observed, *i.e.*, from 3.63 % before laccase treatment to 4.8 % after, indicating that polymerization reactions occurred, depleting the content of catechin, and increasing the content of higher oligomers. Oligomers with more than 3 catechin units were not detected, probably due to the ionization techniques used and/or their precipitation from the reaction mixture.

APE was able to mediate the cross-linking of BLG by laccase. Proteins also became insoluble in the reaction mixture. Highly polymerized BLG could be seen at the top of the running and stacking gel after SDS PAGE. Increasing concentration of APE and ratio of APE: BLG did not increase the amount of the cross-linked protein, although an extensive pellet was obtained due to formation of condensed polyphenols (tannins). The tannin network apparently captures soluble BLG, resulting in its disappearance from the reaction mixture (Fig. 2). In addition, the products of enzymatic conversion of polyphenols were able to inhibit the laccase. Therefore, the BLG cross-linking could not proceed to completion. Similar results were obtained with sour cherry phenols-mediated cross-linking of BLG. A small truncation of the BLG could also be observed, due to the presence of endoprotease in the commercial laccase preparation.<sup>23</sup>

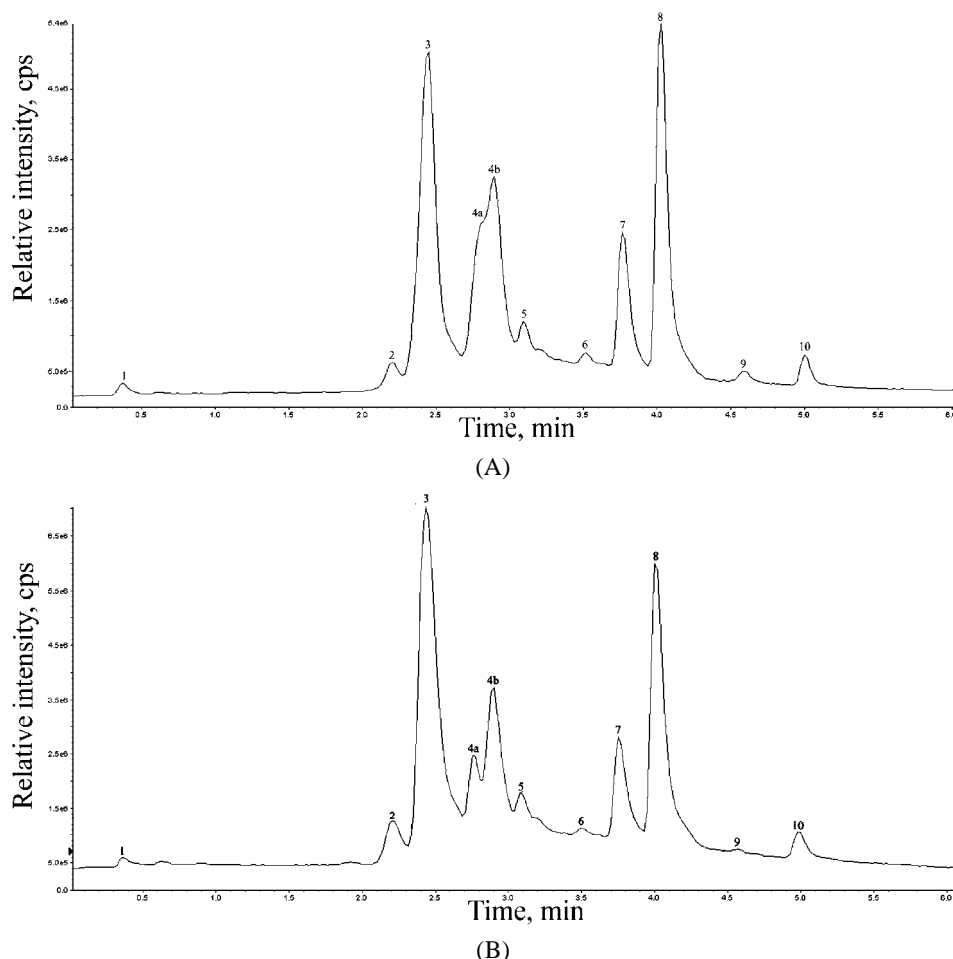


Fig 1. LC-MS chromatograms of apple phenolic extract: A) untreated B) treated with laccase.

TABLE I. The main phenol components of apple phenolic extract untreated and treated with laccase, determined by LC-ESI-MS

Main phenol components	<i>m/z</i>	Peak	Untreated APE		Laccase treated APE	
			<i>t<sub>R</sub></i> / min	% in extract	<i>t<sub>R</sub></i> / min	% in extract
Quinic acid	191	1	0.37	0.56	0.36	0.41
Proanthocyanidin B	577	2	2.20	1.57	2.21	3.42
Chlorogenic acid	353	3	2.44	31.72	2.4	38.19
Neochlorogenic acid	353	4a	2.80	n/a	2.76	5.92
Epicatechin, coumaroylquinic acid	289	4b	2.89	25.74	2.89	14.18
Proanthocyanidin C	865	5	3.09	3.63	3.08	4.81
Proanthocyanidin B, quercetin glucoside	577	6	3.51	0.79	3.50	0.57
Phloretin xyloglucoside	567	7	3.77	8.97	3.75	7.85
Phlorizin	435	8	4.02	23.35	4.00	22.89
Quercetine	301	9	4.59	0.59	4.59	0.31
Phloretin	273	10	5.00	1.63	4.99	1.77

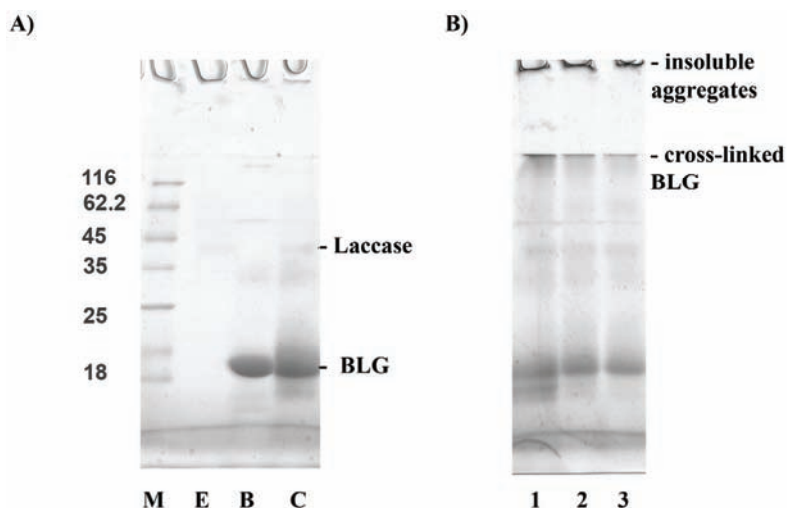


Fig 2. A) Cross-linking of BLG by laccase analyzed by 12 % SDS PAGE; M – molecular markers, E – laccase, B – BLG, C – APE/laccase cross-linked BLG. B) Influence of different concentration of APE on the cross-linking of BLG by laccase: 1 – 0.33 mg mL<sup>-1</sup> GAE APE, 2 – 0.66 mg mL<sup>-1</sup> GAE APE, 3 – 0.99 mg mL<sup>-1</sup> GAE APE.

To test the metabolic prediction for novel proteins, *in vitro* studies with simulated digestive solutions have been widely used. Typically, most food allergens tend to be stable to the peptic and acidic conditions of the digestive system in order to reach and pass through the intestinal mucosa to elicit an allergic response. Therefore, consideration of the resistance of proteins to proteolytic digestion (digestion in simulated gastric or intestinal fluids or by pepsin) has been in-

cluded as one of the components of a comprehensive weight-of-evidence approach to assessing allergenic potential by Codex Alimentarius.<sup>25</sup> Cross-linked BLG was exposed to biphasal digestion by pepsin and pancreatin. Due to insolubility of the cross-linked BLG and the formed tannins, the excess of soluble proteins (laccase, BLG) and soluble polyphenols were removed from reaction mixture by centrifugation and several washing steps before digestion. The control (untreated BLG) was resistant to pepsin digestion, but very susceptible to pancreatin digestion (Fig. 3), as described previously.<sup>26</sup> Cross-linked BLG remained undigested by pancreatin, even after prolonged digestion. The undigested protein remaining in the insoluble fraction of the cross-linked BLG was probably protected from enzyme exposure by a complex network of condensed polyphenols.

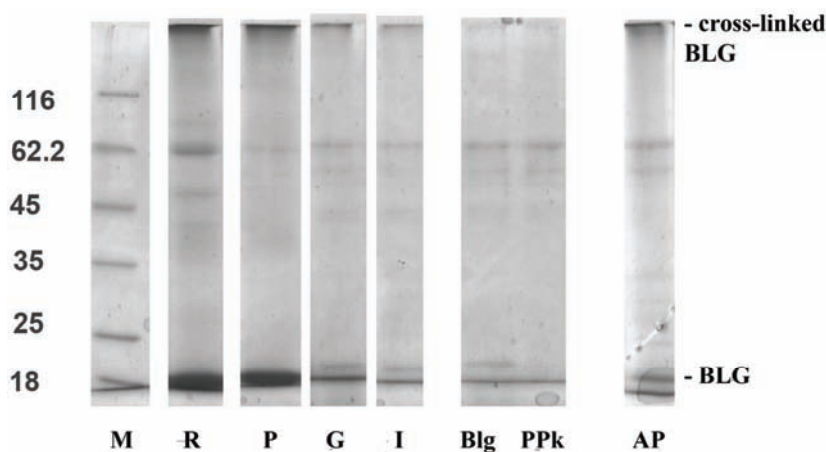


Fig 3. Digestibility of cross-linked BLG by pepsin and pepsin/pancreatin, analyzed by 10 % SDS PAGE; M – molecular markers, R – APE/laccase cross-linked BLG, P – resuspended APE/laccase cross-linked BLG after washing, G – pepsin digestion of APE/laccase cross-linked BLG, I – pepsin/pancreatin digestion of APE/laccase cross-linked BLG, Blg – pepsin/pancreatin digestion of BLG, PPk – control (pepsin and pancreatin), AP – insoluble pellet after pepsin/pancreatin digestion of APE/laccase cross-linked BLG.

It is well known that proteins resistant to both gastric and intestinal digestion are not allergenic. Very recently, the intestinal digestibility of gastric-resistant proteins has been proposed to have implications on oral tolerance to food allergens.<sup>27</sup> An intermediate digestion seems to be required to provide both sensitizing and lack of oral tolerance inducing the capability of a food sensitizer. The data from animal models also show that both gastric and intestinal-resistant allergens do not carry strong food allergy risk or induce oral tolerance, simply because both food allergy and oral tolerance are major histocompatibility complex (MHC) class II-dependent processes and require antigen presentation to the immune system, *i.e.*, readily available peptides in the intestinal fluids.<sup>28</sup> The im-



plications of the present findings are a decreased concentration of intact protein and larger peptides in the intestinal fluids in the case of APE-mediated cross-linking of BLG, leading to lower concentrations of allergen/allergenic peptides in the intestinal fluids. In addition, the anti-nutritive properties of APE-mediated cross-linking of food proteins could find employment in the creation of dietetic preparations, thus helping in the control of obesity and related disorders.

#### CONCLUSIONS

Apple polyphenolic extract is able to mediate enzymatic cross-linking of a major whey allergen,  $\beta$ -lactoglobulin, by laccase. Parallel formation of condensed products (tannins) and a protein-polyphenol network decreased the solubility of BLG and its digestibility by pepsin and pancreatin. The results of the study showed the potential of enzymatic processing in producing new and safer food with improved functional properties.

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#### ИЗВОД

#### ДИГЕСТИБИЛНОСТ $\beta$ -ЛАКТОГЛОБУЛИНА НАКОН ЊЕГОВОГ УНАКРСНОГ ПОВЕЗИВАЊА ДЕЈСТВОМ ЛАКАЗЕ ИЗ *Trametes versicolor* И ПОЛИФЕНОЛА ИЗ ЈАБУКЕ

ZIYAD TANTOUSH, ЛУКА МИХАЈЛОВИЋ, БОЈАНА КРАВИЋ, ЈАНА ОГЉЕНОВИЋ, РАТКО М. ЈАНКОВ, ТАЊА ЋИРКОВИЋ ВЕЛИЧКОВИЋ И ДРАГАНА СТАНИЋ-ВУЧЕНИЋ

*Хемијски факултет, Универзитет у Београду, Студентски брџ 16, 11001 Београд*

$\beta$ -Лактоглобулин (BLG) је важан нутријент млечних производа и важан алерген код алергија на кравље млеко. Циљ ове студије је било испитивање потенцијала лаказе да унакрсно повеже BLG у присуству фенолног екстракта јабуке (APE), као и карактеризација добијених производа са аспекта њихове дигестибилности пепсином и панкреатином. Композиција фенола јабуке коришћених за унакрсно повезивање одређена је помоћу LC-ESI-MS. Фенолни екстракт јабуке садржи знатне количине гликозида кверцетина, катехине и хлорогенску киселину. BLG је унакрсно повезан лаказом у присуству фенола јабуке, при чему је полимеризација учинила BLG нерастворним у реакционој смеси. SDS-PAGE анализа показала је да реакциона смеша садржи хетерогену смешу великих молекулских маса (унакрсно повезан BLG), као и део заосталог мономерног BLG. Ензимско процесовање BLG лаказом, у присуству полифенола јабуке као медијатора, може смањити бифазну пепсин-панкреатинску дигестибилност како мономерног, тако и унакрсно повезаног BLG, и на тај начин смањити његову нутритивну вредност. Такође, смањена дигестибилност BLG може смањити његов алергени потенцијал. Полифеноли јабуке могу се користити за креирање нових, функционалних прехранбених производа, дизајнираних за превенцију како гојазности, тако и болести везаних за преосетљивост.

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